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PRINCIPAL INVESTIGATOR: John Svaren, Ph.D.

CONTRACTING ORGANIZATION: University of Wisconsin
Madison, Wisconsin 53706-1490

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John Svaren, Ph.D.

E-Mail: jpsvaren@wisc.edu

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14. ABSTRACT

Previous work had shown that the EGR1 transactivator is overexpressed in prostate cancer, while expression of its corepressor, NAB2, is reduced. Based on our recent characterization of an interaction between NAB2 and the NuRD (Nucleosome remodeling and disruption) chromatin remodeling complex, we have determined if loss of NAB2 expression results in loss of NuRD targeting to EGR1 target genes. In progress thus far, we have shown that repression of some NAB-regulated target genes in prostate cancer cells requires the NuRD chromatin remodeling complex. We have developed novel chromatin immunoprecipitation assays for the NuRD complex in prostate cells to demonstrate the colocalization of the NuRD complex on EGR1-regulated endogenous target genes. In addition, we have shown that recruitment of the NuRD complex to EGR1 target genes is dependent on NAB2. Finally, NuRD-dependent repression of NAB-regulated repression is sensitive to histone deacetylase inhibitors. These results provide the first functional description of one of the major HDAC-containing chromatin remodeling complexes in prostate cancer cells, and elucidate molecular consequences of loss of NAB2 corepressor function in prostate carcinogenesis by analyzing the mechanism of NAB2 corepressor function.

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INTRODUCTION:

Carcinogenesis in the prostate is often accompanied by overexpression of the EGR1 transcriptional activator, and reduced expression of its corepressor, NAB2 (Abdulkadir et al. 2001). This finding was recently reinforced by a methylation analysis of prostate cancer samples, indicating that the NAB2 promoter is hypermethylated in a majority of prostate cancers (Bastian et al. 2006). Our recent work has shown a novel interaction between NAB2 and the NuRD (Nucleosome Remodeling and Deacetylase) chromatin remodeling complex. The overall hypothesis of this proposal is that reduced expression of NAB2 in prostate cancer prevents NuRD recruitment to EGR1 target promoters, and thereby alters the balance of EGR1 target gene subsets in favor of those promoting growth (i.e. growth factor genes). To address this hypothesis, we will employ dominant negative mutants of CHD4 and NAB2 to determine how they affect expression of endogenous EGR1 target genes. This strategy will be used in several prostate cancer cell lines. In addition, we will use chromatin immunoprecipitation (ChIP) assays to detect NuRD recruitment (and consequent histone deacetylation) in the presence and absence of NAB repression, and will also extend this analysis to surgical samples of prostate carcinoma.

BODY:

Summary of Statement of Work (from original Proposal):

This proposal is designed to test if reduced NAB2 expression results in activation of EGR1 target genes in prostate cancer because of failure to target NuRD activity to these promoters. Dominant negative mutants of CHD4 and NAB2 (as well as RNA interference) will be used to determine how they affect expression of endogenous EGR1 target genes. This strategy will be used to ascertain how NAB/NuRD complexes affect EGR1 activation of growth factor genes, as well as genes involved in growth suppression and apoptosis. Finally, we will also use chromatin immunoprecipitation (ChIP) assays to assay NuRD recruitment and histone acetylation in several EGR1 target promoters.

Summary of Progress (organized by Task)

1. Is the NuRD complex required for repression of endogenous EGR1 target genes by NAB2?

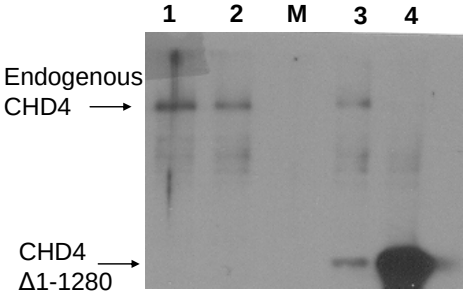
The purpose of this aim was to analyze the connection between NAB repression and the function of the NuRD complex. We are happy to report substantial progress in this task, resulting in a publication in the Journal of Biological Chemistry (see appendix). This publication represents some work that was included as preliminary data for our DOD PCRP grant, as well as further studies since the initiation of the grant cementing the connection between NAB2 and NuRD function (Srinivasan et al. 2006). This publication provides a springboard for further studies outlined in Tasks 1 and 2.

The abstract of this work is as follows:

"Early Growth Response (EGR) transactivators act as critical regulators of several physiological processes, including peripheral nerve myelination and progression of prostate cancer. The NAB1 and NAB2 (NGFI-A/EGR1-binding) transcriptional corepressors directly interact with three EGR family members (Egr1/NGFI-A/zif268, Egr2/Krox20, and Egr3) and repress activation of their target promoters. To understand the molecular mechanisms underlying NAB repression, we found that EGR activity is modulated by at least two repression domains within NAB2, one of which uniquely requires interaction with the CHD4 (Chromodomain Helicase DNA-binding protein 4) subunit of the NuRD (Nucleosome Remodeling and Deacetylase) chromatin remodeling complex. Both NAB proteins can bind either CHD3 or CHD4, indicating that the interaction is conserved among these two protein families. Furthermore, we show that repression of the endogenous *RAD* gene by NAB2 involves interaction with CHD4, and demonstrate colocalization of NAB2 and CHD4 on the *RAD* promoter in myelinating Schwann cells. Finally, the interaction with CHD4 is regulated by alternative splicing of the NAB2 mRNA."

The proposed studies of Task 1 are directed at identifying endogenous target genes that were deregulated by dominant negative NAB2, dominant negative CHD4, as well as by treatment with histone deacetylase inhibitors, which would inhibit the activity of HDAC1 and HDAC2 (histone deacetylase) subunits of the NuRD complex. We prepared recombinant adenovirus using the AdEasy system to express EGR1 and a dominant negative version of CHD4, consisting of a C-terminal fragment of CHD4 that interacts

Dominant Negative CHD4 reduces wild type levels



1: Uninfected

2: AdGFP

3,4: AdCHD4Δ1-1280 (increasing amounts)

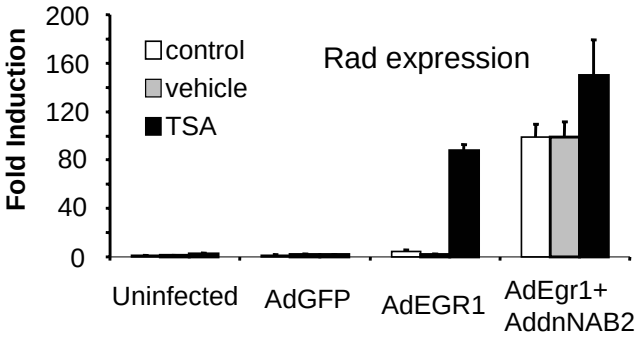
with NAB2, but nonetheless lacks the ATPase domain that is required for NuRD activity. After infection of the M12 metastatic subline of P69SV40T prostate epithelial cells (Bae et al. 1998), Western analysis using an antibody directed against CHD4 revealed that expression of dominant negative CHD4 results in downregulation of the full length endogenous CHD4 protein (see lanes 3 and 4), consistent with results that we have obtained in JEG3 cells (Srinivasan et al. 2006). Based on our studies, we have concluded that expression of the C-terminal fragment results in displacement of endogenous CHD4 from the NuRD complex, rendering it more labile.

As shown in the table, our work has identified several endogenous target genes that are deregulated by the expression of dominant negative NAB2 and CHD4 in the M12 line. The M12 line was infected with the indicated recombinant adenoviruses, and harvested 48 h later for expression analysis. The numbers indicate fold induction of the genes (relative to GFP expressing control), and the standard error is indicated (±). The genes include not only a previously characterized target gene in prostate cancer (e.g. IGF2, insulin-like growth factor 2), but also genes such as Rad, GDF15, GFI1 and HCK. RAD (Ras homolog in diabetes) is a member of the RGK family of GTP binding proteins, which affect Rho-dependent cytoskeletal remodeling (Kelly 2005), and HCK (hemopoietic cell kinase) belongs to the Src family of tyrosine kinases (Lock et al. 1990). GDF15 (growth differentiation factor 15, also known as prostate-derived factor, PDF, NAG1, MIC1, and PLAB) is a member of the TGFβ superfamily, and recent profiling of microdissected tissue has linked GDF15 to prostate carcinogenesis (Cheung et al. 2004). Finally, growth factor independent 1 (GFI1) is an oncogenic repressive transcription factor, as recent work has indicated it might be involved in suppression of naturally occurring 1,25 vitamin D levels in prostate cancer (Dwivedi et al. 2005).

Gene	Ad-EGR1	Ad-dnNab2	Ad-CHD4Δ1-1280	AdEGR1 + Ad-dnNab2	AdEGR1 + Ad-CHD4Δ1-1280
IGF2	1.8 ± 0.1	2.3 ± 1.3	3.8 ± 2.0	**36.5** ± 3.2	**17.9** ± 7.6
RAD	4.1 ± 0.1	3.9 ± 0.06	2.6 ± 0.1	**62.7** ± 7.0	**9.4** ± 0.5
GDF15	1.3 ± 0.9	4.7 ± 1.2	4.8 ± 3.0	**13.3** ± 0.07	**14.9** ± 0.6
Gfi1	0.5 ± 0.4	10 ± 4.0	8 ± 2.4	**8.9** ± 1.0	**16.3** ± 1.4
HCK	0.6 ± 0.02	4.8 ± 0.07	8 ± 0.3	**6.3** ± 0.6	**15.4** ± 2.8

We initially proposed the use of RNA interference as an alternate strategy to address the role of CHD4 in NAB regulation. However, we have limited success in applying RNA interference to prostate cell lines, many of which are not easily transfected. We are continuing to develop this approach, however, and we have also begun to utilize RNA interference against both CHD3 and CHD4 given that there is some evidence that they may be functionally redundant.

Finally, we have utilized two histone deacetylase inhibitors (Trichostatin A and valproic acid), to determine if they affect NAB2 repression. As shown in the graph to the right, TSA greatly enhances induction of the *RAD* gene by EGR1, consistent with the involvement of histone deacetylases associated with the NuRD complex. Interestingly, the derepression of EGR1 activation by expression of dominant negative NAB2 is only minimally affected by exposure to TSA, suggesting that the potentiating effect of TSA is directly related to NAB recruitment of the NuRD complex. Similar results have been obtained with the *IGF2* gene, and derepression of these genes has also been observed with the valproic acid histone deacetylase inhibitor (data not shown).



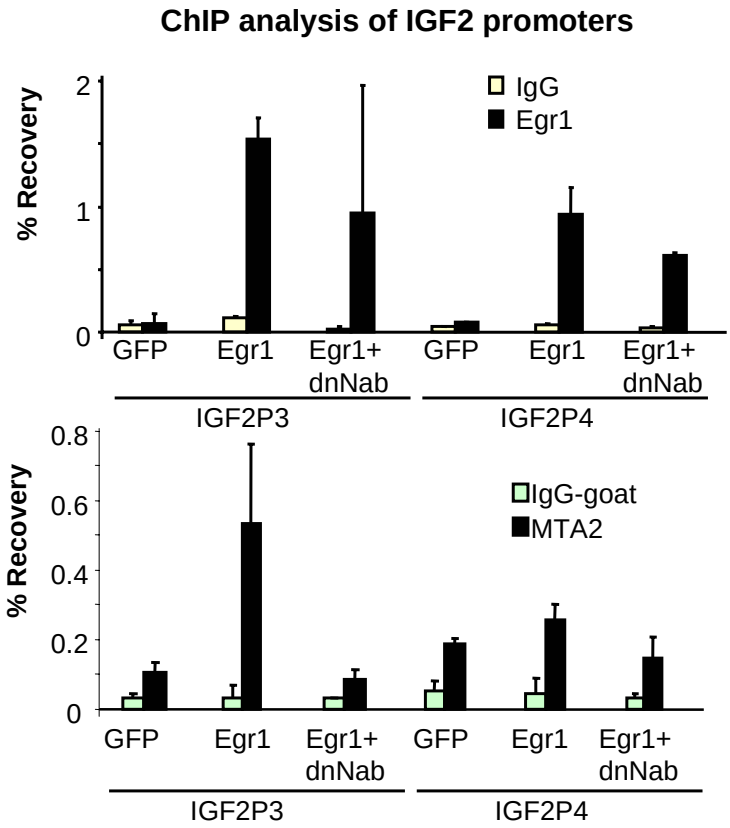
2. Is CHD4 recruited to EGR1 target promoters in a NAB-dependent manner?

This task was directed towards determining if CHD4 and the NuRD complex is recruited to promoters in a NAB-dependent manner. Part of this task involved developing methods to assay recruitment of NuRD complex components of CHD4. We have successfully developed a chromatin immunoprecipitation assay for CHD4 (Srinivasan et al. 2006), and recently showed in this publication (see appendix) that CHD4 is specifically recruited to the NAB-regulated target gene, *RAD*, in a Schwann cell line.

To further test this hypothesis in prostate cells, we have also developed independent chromatin immunoprecipitation assays for another NuRD subunit, MTA2. Use of this assay is also of interest given recent findings that MTA2 (metastastasis-associated protein) is correlated with ER-alpha expression in invasive breast tumors (Cui et al. 2006). To perform chromatin immunoprecipitation assays, M12 cells were treated with

1% formaldehyde to achieve crosslinking. Chromatin was then sonicated and immunoprecipitated with antibodies directed against EGR1, MTA2 or IgG control. The specificity of the assay is tested using purified rabbit (or goat) IgG in a control immunoprecipitation to measure the background of the assay. After reversing the crosslinks, purified DNA was analyzed by quantitative PCR using primers designed to particular regions within the *IGF2* locus. *IGF2* is transcribed from four different promoters; promoters P3 and P4 have multiple EGR1 binding sites, and are also most highly expressed in prostate cells (Svaren et al. 2000). For each primer set, the percent recovery was calculated relative to the input amount of genomic DNA in the immunoprecipitation.

The graph to the right shows ChIP assays of the M12 prostate cell line that is infected with adenoviruses expressing either a) GFP, b) EGR1, or c) EGR1 and dominant negative NAB2. These studies first of all show that binding of MTA2 to the IGF2 P3 promoter is stimulated by EGR1 expression, but this binding is reduced to background levels in the presence of dominant negative NAB2. Similar results are observed in the P4 promoter of IGF2, although the effects are more subtle. Control ChIP assays employing an EGR1 antibody show correlated increased binding of EGR1 upon EGR1 overexpression, but this level of EGR1 binding is relatively unaffected by expression of dominant negative NAB2, as expected. We have observed qualitatively similar results with a CHD4 antibody (not shown), but our recent work has indicated that the MTA2 binding assay is more sensitive, and therefore we have relied on this antibody in recent studies.



We are now extending this analysis to other EGR1 target genes, as well as to analyze levels of histone acetylation as outlined in Task 2. In addition, we are beginning to analyze human prostate tissues using the same techniques. In initial pilot studies, we have successful performed ChIP assays in mouse prostate tissues for EGR1, indicating that ChIP analysis of human tissues is feasible, and we have recently obtained human prostate samples from our collaborator, Dr. David Jarrard for the proposed studies.

3. Does reduced NAB2 expression alter the balance between different subsets of EGR1 target genes?

This task is directed towards identifying if NAB2 alters the balance of EGR1 target gene expression. In this respect, we have identified a number of EGR1 target genes that are not apparently repressed by NAB protein expression. Moreover, our initial hypothesis suggested that these screens would uncover NAB-activated target genes, and our preliminary data indicated that p57 might be a NAB-activated target gene. We have initiated work on this task, and we have obtained some conflicting data in that p57 appeared to be activated by NAB2 in PC3 cells, but we have observed the opposite result in M12 and LAPC4 cells. In addition, we have measured expression of some EGR1 target genes that are growth inhibitory and have seen minimal effects of NAB regulation (e.g. p53, p19/ARF, p73, PTEN, fas ligand).

In order to identify other NAB-activated genes, we performed a candidate screen using the Applied Biosystems 1700 microarray system. In this screen, we identified a few NAB-activated target genes. One of the more interesting ones is CXCL14, which was recently shown to have growth inhibitory effects in prostate cells (Schwarze et al. 2005). We are continuing to screen the cell lines to independently test these findings, as well as identify other novel NAB-regulated genes.

KEY RESEARCH ACCOMPLISHMENTS:

- Identification of novel NAB2-regulated endogenous target genes in prostate cancer cells
- Demonstration that repression of NAB-regulated target genes requires one of the major histone deacetylase-containing complexes: the NuRD chromatin remodeling complex.
- The requirement of the NuRD complex in NAB-mediated repression is sensitive to histone deacetylase inhibitors.
- Development of novel chromatin immunoprecipitation assays for the NuRD complex in prostate cells to demonstrate the colocalization of the NuRD complex on EGR1-regulated endogenous target genes.
- Recruitment of the NuRD complex to EGR1 target genes is dependent on NAB2.
- First functional description of one of the major HDAC-containing chromatin remodeling complexes in prostate cancer cells.
- The analysis provide the first analysis of the mechanism of NAB2 corepressor function in prostate cells
- Elucidating molecular consequences of loss of NAB2 corepressor function in prostate carcinogenesis.

REPORTABLE OUTCOMES:

See appendix for copies of the following

<u>One manuscript has been published as a result of the work outlined above:</u>

Srinivasan, R., Mager, G.M., Ward, R.M., Mayer, J., and Svaren, J. (2006) The NAB2 corepressor interacts with the CHD4 subunit of the NuRD complex, **Journal of Biological Chemistry** 281:15129-15137

<u>One abstract submitted for presentation at the annual meeting of the American Association of Cancer Research (4/07):</u>

Srinivasan, R. and Svaren, J. The NAB2 corepressor targets the NuRD chromatin remodeling complex to EGR1 target genes in prostate

CONCLUSION:

One of the major unexplored frontiers in understanding altered gene expression in prostate cancer is the elucidation of epigenetic effects caused by chromatin remodeling machinery. Advances in this area have already lead to clinical trials using entirely new strategies employing histone deacetylase (HDAC) inhibitors to treat some types of cancer (Drummond et al. 2004; Zelent et al. 2004). One of the principal complexes targeted in such strategies is the NuRD (Nucleosome Remodeling and Deacetylase) complex, which contains two molecules of histone deacetylases as integral components. Although initial results from such studies are promising, drugs that inhibit histone deacetylases could have manifold effects, not all of which may ultimately be beneficiary. In order to refine therapies of this type, it will be necessary to probe how various chromatin remodeling complexes are altered in prostate cancer and how they might be affected by drugs that inhibit histone deacetylases.

In conclusion, we have accomplished several of the tasks outlined in the proposal that we submitted. First of all, we have now demonstrated that several endogenous target genes are functionally repressed by NAB corepressors in prostate cells, and this repression is dependent on the activity of the NuRD chromatin remodeling complex, consistent with our recently described interaction of NAB2 with the CHD4 subunit of the NuRD complex. Furthermore, this interaction has been independently tested by determining that NAB repression of specific EGR1 target genes is dependent on histone deacetylase activity, presumably derived from the HDAC1 and HDAC2 subunits of the NuRD complex. To establish the mechanism of these effects on the transcriptional templates, we have successfully coupled expression analysis with novel chromatin immunoprecipitation assays that demonstrate binding of EGR1 and components of the NuRD complex to regulatory regions of EGR1 target genes. These studies now make it possible to extend our analysis to human prostate cancer samples as outlined in our proposal.

Given recent work that NAB2 expression is lost in a majority of human prostate cancer samples, presumably through hypermethylation of the NAB2 promoter (Bastian et al. 2006), it is important to understand the functional consequences of losing this potentially growth-regulatory pathway. This may enable therapeutic approaches to either restore NAB2 function in some manner to restore the balance of EGR1-regulated target genes. Given a wealth of evidence indicating that EGR1 is an important regulator of prostate carcinogenesis, this molecular information will illuminate its role and pinpoint biochemical steps that can be targeted to prevent and treat prostate cancer.

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Srinivasan R., Mager G. M., Ward R. M., Mayer J. and Svaren J. (2006) NAB2 represses transcription by interacting with the CHD4 subunit of the NuRD complex. *J Biol Chem* **281,** 15129-15137.

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APPENDICES:

Srinivasan, R., Mager, G.M., Ward, R.M., Mayer, J., and Svaren, J. (2006) The NAB2 corepressor interacts with the CHD4 subunit of the NuRD complex, **Journal of Biological Chemistry** 281:15129-15137

One abstract accepted for presentation at American Association of Cancer Research meeting (4/07):

Srinivasan, R. and Svaren, J. The NAB2 corepressor targets the NuRD chromatin remodeling complex to EGR1 target genes in prostate

Abstract for Annual Meeting of the American Association for Cancer Research (4/07)

The NAB2 corepressor targets the NuRD chromatin remodeling complex to EGR1 target genes in prostate.

Rajini Srinivasan, Rebecca M. Ward, and John Svaren
Department of Comparative Biosciences, University of Wisconsin-Madison

The goal of these studies is to determine how loss of the NAB2 (NGF1-A binding protein 2) corepressor in prostate cancer affects gene regulation and chromatin structure of EGR1 (Early growth Response) target genes. EGR1 is a transcription factor that is overexpressed in prostate cancer and progression of prostate cancer in mouse models is severely compromised in the absence of EGR1. NAB corepressors directly interact with EGR1 and are important regulators of EGR1 activity. Interestingly, while EGR1 is overexpressed in prostate cancer, NAB2 expression is low or absent in majority of prostate cancer samples, including both PIN (Prostate Intraepithelial Neoplasia) lesions and metastases. The role of EGR1 in prostate cancer progression is complex as it regulates both pro-proliferative genes, as well as genes involved in apoptotic/senescence pathways. Our analysis indicates that loss of NAB2 expression alters the balance of EGR1 target genes in favor of activation of genes involved in growth promotion. Moreover, we have recently shown that NAB2 represses EGR target genes through its association with Chromodomain Helicase DNA Binding Protein 4 (CHD4), which is a component of the Nucleosome Remodeling and Deacetylase (NuRD) complex. The NuRD complex is one of the major histone deacetylase (HDAC)-containing complexes in the nucleus and also incorporates Metastasis-Associated Factor 1/2 (MTA1/2) as integral components. Using microarray and chromatin immunoprecipitation analyses, we have identified NAB-regulated target genes in prostate cell lines and demonstrated that EGR1, NAB2 and subunits of the NuRD complex are directly recruited to several target promoters. Many of the same genes are also derepressed by exposure to histone deacetylase inhibitors, indicating that the HDAC subunits of the NuRD complex play an important role in NAB regulation. Overall, these data indicate that loss of NAB2 results in failure to target the repressive NuRD complex to specific EGR1 target genes in prostate cancer cells.

This work was supported by a grant from the Department of Defense Prostate Cancer Research Program.

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NAB2 Represses Transcription by Interacting with the CHD4 Subunit of the Nucleosome Remodeling and Deacetylase (NuRD) Complex*

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Rajini Srinivasan[‡1], **Gennifer M. Mager**[§1], **Rebecca M. Ward**[‡], **Joshua Mayer**[¶], **and John Svaren**[‡§¶2]

From the [‡]*Department of Comparative Biosciences, the* [§]*Molecular and Cellular Pharmacology Training Program, and the* [¶]*Cellular and Molecular Biology Training Program, University of Wisconsin, Madison, Wisconsin 53706*

Early growth response (EGR) transactivators act as critical regulators of several physiological processes, including peripheral nerve myelination and progression of prostate cancer. The NAB1 and NAB2 (NGFI-A/EGR1-binding protein) transcriptional corepressors directly interact with three EGR family members (Egr1/NGFI-A/zif268, Egr2/Krox20, and Egr3) and repress activation of their target promoters. To understand the molecular mechanisms underlying NAB repression, we found that EGR activity is modulated by at least two repression domains within NAB2, one of which uniquely requires interaction with the CHD4 (chromodomain helicase DNA-binding protein 4) subunit of the NuRD (nucleosome remodeling and deacetylase) chromatin remodeling complex. Both NAB proteins can bind either CHD3 or CHD4, indicating that the interaction is conserved among these two protein families. Furthermore, we show that repression of the endogenous *Rad* gene by NAB2 involves interaction with CHD4 and demonstrate colocalization of NAB2 and CHD4 on the *Rad* promoter in myelinating Schwann cells. Finally, we show that interaction with CHD4 is regulated by alternative splicing of the NAB2 mRNA.

By virtue of their ability to regulate the early growth response (EGR)[3] family of transactivators, NAB (NGFI-A-binding protein) corepressors play an important role in regulating inflammation, nervous system function, and prostate cancer development. The NAB1 and NAB2 corepressors interact with a conserved domain found within Egr1 (also called NGFI-A/zif268), Egr2/Krox20, and Egr3 (1–3). The remaining family member, Egr4/NGFI-C, shares substantial homology with other EGR family members but lacks the NAB interaction domain and is therefore resistant to NAB repression.

Members of the EGR family play diverse physiological roles, including having both positive and negative effects on growth. For example, Egr1 null fibroblasts bypass senescence because of reduced expression of the *p53* gene (4). On the other hand, EGR1 overexpression is also involved in the development of prostate cancer (5, 6), as it regulates several growth factor genes (7–10). The other EGR family member that has been studied intensively is Egr2/Krox20. Targeted disruption of the mouse *Egr2* gene resulted in defects in hindbrain segmentation, bone development, and peripheral nerve myelination by Schwann cells (11–14). A number of Egr2 target genes in the hindbrain and Schwann cells have been identified, including several Hox family members, EphA4, and myelin-associated genes such as myelin protein zero and myelin basic protein (13, 15–21).

Several experiments in various systems have established that NAB corepressors are important regulators of EGR activity. NAB1 and NAB2 both repress EGR activation of several promoters (3, 22–26). Interestingly, although EGR1 is overexpressed in prostate cancer (5, 6), NAB2 expression is reduced in a majority of prostate cancer samples (27). This observation is consistent with the idea that derepression of EGR1 activity is a progression factor in prostate cancer. The importance of NAB regulation is underscored by the identification of a recessive mutation in the NAB-binding domain of EGR2 (I268N) in a family affected with an inherited form of congenital hypomyelinating neuropathy (28, 29). Congenital hypomyelinating neuropathy resembles the non-myelinating phenotype of the peripheral nervous system observed in Egr2/Krox20-deficient mice (13). Similarly, a double knock-out of the *NAB1* and *NAB2* genes causes early lethality and impaired myelination (30), indicating that NAB corepressors are required for peripheral nerve myelination.

Although diverse physiological data have demonstrated that NAB corepressors play a critical role in regulation of EGR activity, the molecular mechanism by which these corepressors act has remained elusive. NAB1 and NAB2 share a high degree of homology (2) and are able to homo- and heteromultimerize with each other (22). NAB1 and NAB2 are nuclear proteins, and they repress when tethered to active promoters by fusion to a Gal4 DNA-binding domain (DBD) (31). Therefore, we hypothesized that NAB proteins recruit other proteins in order to regulate EGR activity. We now show that the C-terminal domain of NAB2 interacts with the chromodomain helicase DNA-binding protein 4 (CHD4) subunit of the nucleosome remodeling and deacetylase (NuRD) complex and that this interaction is required for repression by this domain.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—The yeast two-hybrid screen (32) was performed in the Molecular Interaction Facility, University of Wisconsin Biotechnology Center. Mouse embryonic and brain libraries in pGAD-T7Rec (BD Biosciences) were screened with a construct containing amino acids 130–525 of the NAB2 protein fused to the GAL4 DNA-binding domain in pBUTE (a kanamycin-resistant version of GAL4 bait vector pGBDUC1). Approximately 18 million clones were screened via

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This work is dedicated with gratitude to the memory of Wolfram Hörz (1944–2005).
[1] These authors contributed equally to this work.
[2] To whom correspondence should be addressed: 2015 Linden Dr., School of Veterinary Medicine, Madison, WI 53706. Tel.: 608-263-4246; Fax: 608-263-3926; E-mail: jpsvaren@wisc.edu.
[3] The abbreviations used are: EGR, early growth response; CHD, chromodomain helicase DNA-binding (protein); NCD, NAB conserved domain; CID, CHD4-interacting domain; HDAC, histone deacetylase; RAD, Ras homolog in diabetes; PHD, plant homeodomain; siRNA, short interfering RNA; DBD, DNA-binding domain; TSA, trichostatin A; HA, hemagglutinin; NuRD, nucleosome remodeling and deacetylase (domain); P11, postnatal day 11; RT, reverse transcription.

mating in yeast strain PJ694. After isolation of prey plasmids from positive pools, 18 plasmids were positive after retransformation into the bait-containing strain. Of these, two contained clones of mouse CHD4.

Plasmids—Segments of the CHD4 (containing amino acids 1281–1915) and CHD3-(Δ1–1311) genes were cloned in-frame with an N-terminal 3× FLAG epitope in the pcDNA3.1 vector (Invitrogen). Human HA-NAB2 (containing amino acids 34–525) was generated by introducing a C-terminal HA epitope. Deletion of amino acids 251 to 353 in HA-NAB2 was used to create NAB2ΔNCD2 (NCD1+CID), and NAB2ΔCID (NCD1+NCD2) lacks amino acids 386–525. The last 18 amino acids are excluded in the construct NAB2Δ507–525. The CID construct consists of amino acids 357–525, including the nuclear localization sequence to permit nuclear translocation. For NAB2 constructs lacking NCD1, translation initiation occurred at Met-141. A naturally occurring splice variant of NAB2 lacks amino acids 426–489 (exon 6 of NAB2). The indicated NAB2 segments were fused to the Gal4 DBD in pM1 (33).

The altered specificity version of Egr2 was generated by mutagenesis of the second zinc finger from SRSDHLTTHIR to SQSVHLQSHSR, as described for Egr1 (34). The corresponding reporter plasmid was created by inserting nine repeats of an altered EGR2 binding site (GCGTGAGCG) into the pGL2 vector (Promega) containing the adenovirus E1B TATA element. For mammalian two-hybrid experiments, amino acids 1281–1915 of CHD4 were fused to the Gal4 DNA-binding domain in the pM1 vector. The NAB2VP16 construct was created by fusing the VP16 activation domain to the C terminus of NAB2, as described for the NAB1-VP16 construct (31). Constructs for NAB1ΔNCD1-(Δ2–210), the luciferase reporter containing the thymidine kinase promoter with five upstream Gal4 binding sites, and the Gal4 reporter containing a minimal TATA element have been described previously (31).

Coimmunoprecipitation Analysis—QT6 (Quail fibroblast) or 293T cells were cultured as described previously (35), plated at a density of 5×10^5 cells/ml, and transfected using LT-1 (Mirus) transfection reagent according to manufacturer's protocol. Bluescript plasmid (Stratagene) was included as needed to make a total of 2 μg/well/6-well plate. After 48 h, cells were washed once in phosphate-buffered saline and extracted with lysis buffer containing 6% glycerol, 20 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.1% Nonidet P-40, and 200 mM NaCl, with the addition of Complete Mini protease inhibitors (Roche Applied Science) for 10 min at room temperature. The lysates were centrifuged at 10,000 × *g* for 15 min at 4° C. 50 μl (or 200 μl; see Fig. 2*C*) of the supernatant was mixed with 25 μl of anti-HA rat monoclonal affinity matrix (Roche Applied Science) in lysis buffer containing 100 mM NaCl (final concentration). After incubation for 2 h at room temperature on a rocking platform, the matrix was washed five times with 250 μl of binding buffer. The reciprocal immunoprecipitation used M2 anti-FLAG-agarose beads (Sigma) according to the manufacturer's recommendations. After the final wash, proteins were eluted by boiling for 2 min in 1× Laemmli buffer prior to immunoblotting using antibodies directed against the FLAG epitope (M2, Sigma), HA epitope (rat monoclonal from Roche Applied Science or rabbit polyclonal from Sigma), or CHD3/4 (BD Biosciences 611846). Other antibodies used were: α-tubulin (Santa Cruz sc-5546), CHD3/4 (Santa Cruz sc-11378), and Egr2 (Covance PRB236P). Membranes were incubated with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and detected using West Pico or West Dura chemiluminescence detection reagents (Pierce).

Cell Culture and Transfections—For reporter assays, JEG3 cells (human trophoblast cell line) were cultured in minimal essential medium supplemented with 5% bovine growth serum. Transfections were carried out in duplicate in 12-well plates seeded with 3×10^4 cells/well. Unless otherwise indicated, cells were transfected with 250 ng of the indicated luciferase reporter plasmid, 100 ng of a LacZ reporter driven by a cytomegalovirus promoter, the indicated expression plasmids, and pBluescript as required to make a total of 1 μg of DNA/well using LT-1 transfection reagent (Mirus). After 48 h, cells were harvested, and the level of luciferase activity was measured and normalized to β-galactosidase activity, which was measured using the GalactoLight Plus kit (Applied Biosystems).

A custom SMARTpool mixture of four short interfering RNAs (siRNAs) directed against human CHD4 and the control siRNA (siCONTROL nontargeting siRNA 1) were purchased from Dharmacon. JEG3 or 293 cells were transfected with 62.5 pM siRNA using Lipofectamine 2000 as recommended by the manufacturer (Invitrogen). For reporter assays in the presence of siRNA, JEG3 cells were simultaneously transfected with siRNA and the plasmids described above using Lipofectamine 2000. Luciferase and β-galactosidase assays were then carried out 48 h later as described above.

Primary rat Schwann cells were cultured and infected as described (19) using recombinant adenoviruses prepared using the AdEasy system (36). After 48 h, purified RNA was analyzed by quantitative RT-PCR using SYBR Green dye as described (8) on a TaqMan 7000 sequence detection system (Applied Biosystems). Relative amounts of the human and rat *RAD* genes were determined using the Comparative Ct method (37) and normalized to the relative levels of 18 S rRNA. Primer sequences are available upon request.

PCR—Primers flanking the exon 6 region of NAB2 (GGTTGGAGAACAGAGTCACAATGA and GGCAGCGGTCCAGCAA) were used to amplify the full-length and alternately spliced forms of NAB2 from cDNA prepared from various mouse tissues.

Chromatin Immunoprecipitation Assays—All experiments were performed in strict accordance with experimental protocols approved by the University of Wisconsin School of Veterinary Medicine. After euthanasia of Sprague-Dawley rat pups at postnatal day 11 (P11), sciatic nerves were dissected (pooled from seven pups) and immediately minced in phosphate-buffered saline containing 1% formaldehyde for 25 min at room temperature. Nerves were washed in phosphate-buffered saline, resuspended in 150 mM NaCl, 10% glycerol, 50 mM Tris, pH 8.0 (with a 1:1000 dilution of Sigma protease inhibitor mixture), and homogenized using the Tissue Tearor (biospec). Triton X-100 was added to 0.3%, and the lysate was sonicated in the presence of 100 mg of glass beads, alternating 10-s pulses with 50 s of cooling for a total of 20 min. Sonicated chromatin (containing 300 μg of protein as determined by the Bio-Rad protein assay) was used for each immunoprecipitation, and 10% of this amount was saved as an input. Lysates were incubated with 2 μg of anti-Egr2/Krox20 (Covance), anti-Nab2 (Santa Cruz), anti-CHD3/4 rabbit polyclonal (Santa Cruz sc-11378), or normal rabbit IgG (Upstate) control antibody. Immune complexes were collected with 25 μl of a protein G-agarose slurry (Pierce) blocked with herring sperm DNA (Fisher) and 0.5 mg of bovine serum albumin. Beads were washed in low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris 8.1, 150 mM NaCl) and high salt buffer (same buffer containing 500 mM NaCl) followed by 0.25 M LiCl, 1% IGEPAL, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris, pH 8.1. All buffers contained a 1:1000 dilution of Sigma Protease inhibitor mixture. Complexes were eluted with 1% SDS, 0.1 M $NaHCO_3$, and 200 mM NaCl. Cross-links were reversed by heating at 65 °C for 5 h, and DNA was purified using the QIAquick PCR purification kit (Qiagen). Quantitative PCR was performed on the same samples in duplicate. Values are expressed as percent recovery relative to the input DNA. Sequence analysis of the *Rad* gene identified potential Egr2 sites conforming to the previously defined consensus Egr2 binding site (38). Primers used for analysis are: Rad -1470, ACCCCCACCACAGT-

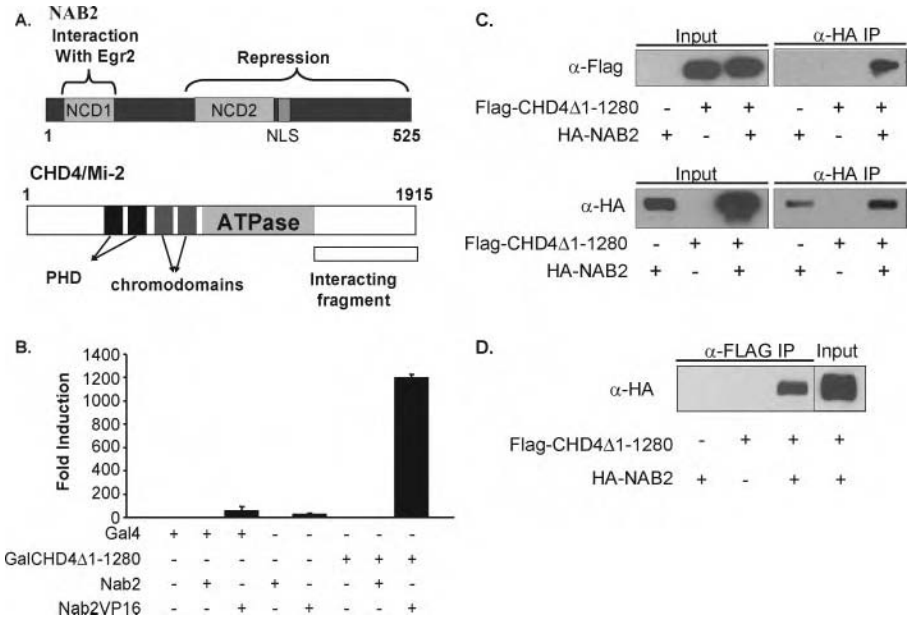

FIGURE 1. **The NAB2 repression domain interacts with CHD4.** *A*, the NAB2 diagram shows two domains that share high homology with NAB1 (NAB conserved domains). NCD1, NCD2, and the nuclear localization sequence (*NLS*) encompass amino acids 34–129, 245–356, and 361–379, respectively. A segment of NAB2 encompassing the repression domain (amino acids 130–525) was used to screen mouse brain and embryo libraries, and the yeast two-hybrid screen identified two independent clones containing the C-terminal end of CHD4 (beginning at amino acids 1281 and 1290, respectively). The CHD4 diagram indicates the conserved chromodomains, PHD zinc finger, and ATPase domains. *B*, for the mammalian two-hybrid assay, JEG3 cells were transfected with a luciferase reporter (containing five Gal4 binding sites) along with 40 ng of CHD4Δ1–1280 tethered to the Gal4 DNA-binding domain in pM1 vector and/or 40 ng NAB2 fused to the VP16 activation domain, as indicated. As negative controls, cells were transfected with pM1 alone (Gal4) and NAB2. Results are normalized to the luciferase activity of the reporter plasmid alone. Means ± S.D. of two replicate experiments are shown. *C*, QT6 cells were transfected with 1 μg of 3× FLAG-tagged CHD4Δ1–1280 and/or 1 μg of HA-NAB2 as indicated. Lysates of the transfected cells were prepared and immunoprecipitated with anti-HA. Immunoprecipitates were immunoblotted with an anti-FLAG antibody to assess interaction (*top panel*). The *Input lanes* represent 4% of the amount of lysate used for the immunoprecipitation, and all lanes are from the same exposure. As a control, inputs (40% of total) and immunoprecipitates were also immunoblotted with anti-HA rabbit polyclonal antibody (*bottom panel*). All lanes are taken from the same exposure. *D*, the same lysates as in *C* were analyzed by immunoprecipitating CHD4Δ1–1280 with anti-FLAG antibody. Immunoprecipitates were immunoblotted with anti-HA antibody to assess interaction. The *Input lane* represents 40% of the amount of lysate used in the immunoprecipitation, and all lanes are from the same exposure.

CATTGT and CTTTGGGACAGGAACTTGCTCT; Rad −130, GGGTAAGGGCTGGTAGAGGTTT and CGCTGGATCGCGGTTCT; IMG2a, GAAATTCTGCCCTGCACTTCC and GCTTTGCATTGAGGGAGGATC.

RESULTS

CHD4 Interacts with NAB2—NAB1 and NAB2 contain two highly conserved domains called NAB conserved domains 1 and 2 (NCD1 and NCD2). NCD1 is necessary for interaction with EGR proteins and is also required for multimerization of NAB proteins (2, 22). Repression by NAB1 was shown to require NCD2 as well as other regions near the C terminus (31). To identify interacting proteins that might mediate repression by NAB2, we employed a yeast two-hybrid screen of mouse brain and embryonic libraries for proteins that interact with amino acids 130–525 of NAB2. This screen identified two independent clones of a C-terminal portion of CHD4 (Fig. 1*A*). CHD4 is an ATP-dependent, nucleosome remodeling subunit of the NuRD complex, which has been shown to repress promoters to which it is targeted (reviewed in Refs. 39–41). None of the other interacting clones encoded NuRD subunits.

Additional evidence for NAB2/CHD4 interaction was provided by a mammalian two-hybrid analysis (Fig. 1*B*), in which the CHD4 C terminus was fused to the Gal4 DBD and NAB2 was fused to the VP16 activation domain. Cotransfection of these two constructs with a luciferase reporter containing Gal4 binding sites resulted in a significant increase in luciferase activity compared with transfection of either construct alone. The potential interaction between CHD4 and NAB2 was tested independently *in vivo* using a coimmunoprecipitation assay with epitope-tagged versions of NAB2 and the CHD4 C terminus (CHD4Δ1–1280). The CHD4 construct was immunoprecipitated by the anti-HA antibody only in the presence of the HA-tagged NAB2 (Fig. 1*C*). Conversely, co-immunoprecipitation of NAB2 by anti-FLAG was dependent on expression of FLAG-tagged CHD4 (Fig. 1*D*).

Identification of the CHD4-interacting Domain of NAB2—Deletion analysis of NAB1 implicated the NCD2 domain and the extreme C terminus in transcriptional repression (31). Based on these studies, HA-tagged versions of NAB2 were generated that contained a deletion of the NCD2 domain, the C terminus (amino acids 386–525), or the C-terminal 18 amino acids (Fig. 2*A*). To eliminate the possibility that any of these proteins might interact indirectly with CHD4 as a result of multimerizing with endogenous NAB proteins, the NCD1 domain was deleted from these constructs. Interestingly, analysis of these mutant proteins revealed that the NCD2 domain was not essential for interaction with CHD4 (Fig. 2*B*). Deletion of the 18 amino acids at the C terminus of the NAB2 protein also failed to disrupt interaction with CHD4. However, removal of amino acids 386–525 of the NAB2 protein prevented interaction with CHD4. Furthermore, expression of amino acids 386–525, together with the nuclear localization signal, was sufficient for interaction with CHD4. This C-terminal region of NAB2 was therefore designated as the CID (CHD4-interacting domain). We also tested whether NAB2 could interact with endogenous CHD4 by transfecting cells with the indicated NAB2 constructs in the absence of transfected CHD4. After immunoprecipitation of NAB2 with anti-HA, we were able to detect associated CHD3/CHD4, which was not observed using a NAB2 construct in which the CID was deleted (Fig. 2*C*).

NAB/CHD Interaction Is Conserved among Family Members—The antibody used in the endogenous assay in Fig. 2*C* detects both CHD3 and CHD4. CHD4 shares several regions of homology with CHD3, including not only the ATPase, PHD, and chromodomains but also

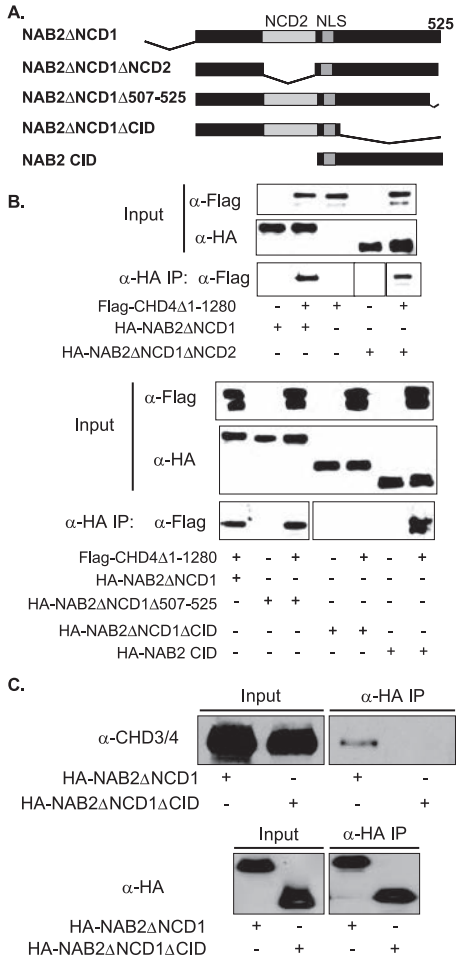

FIGURE 2. **Identification of CHD4-interacting domain of NAB2.** *A*, each of the indicated NAB2 deletion constructs contained the HA epitope and the nuclear localization sequence (*NLS*) to allow translocation into the nucleus. *B*, QT6 cells were transfected with 1 μg of each of the deletion constructs of NAB2 along with 1 μg of 3× FLAG-tagged CHD4Δ1–1280 as indicated. Lysates were immunoprecipitated with anti-HA, and bound proteins were immunoblotted with anti-FLAG antibody to test for interaction. Immunoblotting analysis with anti-FLAG (4% input) and anti-HA (40% input) confirmed that the CHD4 and Nab2 constructs were expressed in the indicated lysates. All lanes within a given panel were taken from the same exposure. *C*, 293T cells were transfected with 10 μg of either HA-NAB2ΔNCD1 or HA-NAB2ΔNCD1ΔCID as indicated. Lysates were immunoprecipitated with anti-HA antibody, and CHD proteins were detected using an anti-CHD3/4 antibody (*top panel*); *Input lanes* represent 35% of the protein in the immunoprecipitation (*IP*) lanes. Inputs and immunoprecipitates were also probed with an anti-HA antibody (*bottom panel*); *Input lanes* represent 17.5% of the protein in the immunoprecipitation lanes. All lanes within a given panel were taken from the same exposure.

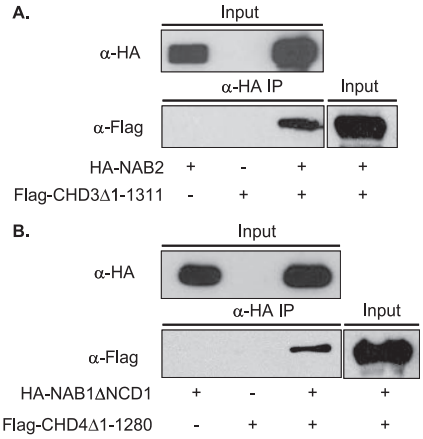

FIGURE 3. **NAB and CHD family members share capacity for interaction.** QT6 cells were transfected as indicated with 1 μg of 3× FLAG-tagged CHD3Δ1–1311 and/or HA-NAB2 (*A*) or 1 μg of 3× FLAG-tagged CHD4Δ1–1280 and/or 1 μg of HA-NAB1ΔNCD1 (*B*). Immunoprecipitations with the anti-HA antibody (*IP*) were blotted with an anti-FLAG antibody. Expression of the indicated proteins in the lysates was confirmed by immunoblotting with anti-FLAG and anti-HA. *Input lanes* represent 4% (*Flag panel*) and 40% (*HA panel*) of the amount of lysate used in the immunoprecipitation, and all lanes are from the same exposure.

other regions within the C terminus (42). In addition, CHD3 has been found in NuRD-like complexes (43–45), suggesting that its molecular role is at least partially redundant to that of CHD4. To test a potential interaction of CHD3 with NAB2, a comparable portion of the C-terminal domain of CHD3 (CHD3Δ1–1311) was epitope-tagged and found to associate with NAB2 (Fig. 3*A*) in a coimmunoprecipitation assay. In addition, NAB1 and NAB2 share a considerable degree of homology, and in functional assays, we have never observed any substantial differences in their ability to repress EGR-mediated transcription (2, 3). As shown in Fig. 3*B*, NAB1 can also interact with CHD4, suggesting that NAB1 and NAB2 share the capacity to repress transcription by interacting with CHD3 and CHD4.

Disruption of NAB2 Function by Dominant Negative CHD4 Constructs—There have been several biochemical characterizations of mammalian NuRD complexes, but none of these studies has identified NAB2 as a stably associated subunit (43, 44, 46–48). Accordingly, the observed coimmunoprecipitation of transfected NAB2 and CHD4 might reflect a more transient association involved in NuRD recruitment to EGR target genes rather than a stable complex. Therefore, we proceeded to test whether endogenous CHD4 is a functional requirement for repression by NAB2.

Dominant negative CHD4 mutants were used to test the involvement of CHD4 in repression by NAB2. We tested whether expression of the CHD4 C terminus (CHD4Δ1–1280) could interfere with NAB repression in a dominant negative manner, because it binds to NAB2 but lacks the ATPase and other domains required for CHD4 function. In addition, we created a full-length dominant negative CHD4 protein by mutating a conserved lysine (Lys-750) in the ATPase domain (referred to as CHD4K750C). Analogous mutations have been successfully used to create dominant negatives to study ATPase-dependent chromatin remodeling complexes in yeast, *Drosophila*, and mammalian systems (49–51). A similar CHD4 mutant has been used to test the involvement of CHD4 in repression of methylated DNA (52). As expected, the K750C mutation did not affect the ability of CHD4 to associate with NAB2, as assessed by coimmunoprecipitation (data not shown).

We evaluated the ability of the two CHD4 mutant constructs to affect NAB2 repression using an Egr2-dependent reporter assay. To provide an assay with sufficient dynamic range to study NAB repression of EGR activity, we generated an altered specificity mutant of Egr2 (Alt. Egr2) that is analogous to one described previously for Egr1 (34). This mutant, containing four point mutations in the second zinc finger, activates through a variant EGR binding site that is not bound efficiently by endogenous EGR proteins, allowing much greater activation of the reporter. In this altered specificity system, NAB2 represses Egr2 activity down to basal levels (Fig. 4). Cotransfection with either CHD4 mutant partially alleviated this repression, suggesting that CHD4 is required for repression by NAB2. Several controls tested the specificity of the mutant CHD4 constructs. First, expression of wild type CHD4 had little effect on repression. Second, the CHD4 mutants were also tested with an Alt. Egr2 construct containing the I268N mutation, which abrogates the ability of NAB proteins to bind and repress Egr2 activity (29). Importantly, expression of the mutant CHD4 constructs did not alter the activity of Alt. Egr2 containing the I268N mutation (data not shown),

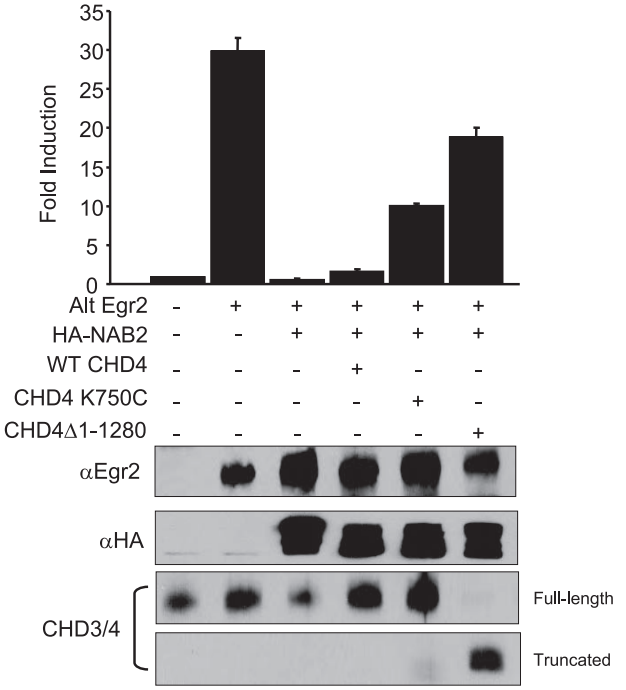

FIGURE 4. **Dominant negative CHD4 constructs interfere with NAB2 repression.** JEG3 cells were transfected with an altered (*Alt*) Egr2 reporter (containing nine altered Egr2 binding sites, GCGTGAGCG) and 20 ng of altered Egr2. Where indicated, cells were transfected with 20 ng of wild type NAB2 and 200 ng of wild type (*WT*) CHD4, CHD4K750C, or CHD4Δ1–1280 expression plasmids. Results are normalized to the luciferase activity of the reporter plasmid alone. Means ± S.D. of two replicates are shown. Immunoblotting was carried out on the other set of replicates to confirm expression of transfected constructs. For each antibody, all lanes are from the same gel and exposure.

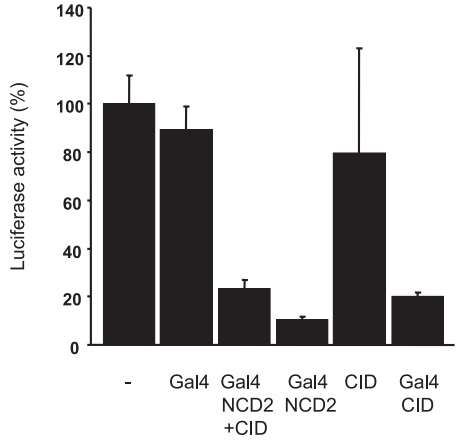

FIGURE 5. **NAB2 has two independent repression domains.** JEG3 cells were transfected with a luciferase reporter plasmid driven by a constitutively active thymidine kinase promoter containing Gal4 sites together with 100 ng of one of the indicated constructs tethered to the Gal4 DBD. NCD2+CID contains amino acids 130–525 of NAB2, NCD2 consists of amino acids 245–362, and CID contains amino acids 357–525. The unfused CID and the Gal4 DBD were included as negative controls. The luciferase activity of the reporter alone was set at 100%. Means ± S.D. of two replicates are shown.

indicating that the derepression of Egr2 activity caused by mutant CHD4 proteins is NAB-dependent. Finally, expression of the mutant constructs did not alter reporter activity in the absence of Egr2 (data not shown), and therefore we concluded that both CHD4 mutants specifically affect NAB repression in a dominant negative manner.

Blotting of lysates from cells transfected in parallel indicated that the CHD4 constructs did not affect expression levels of Egr2 or NAB2. Interestingly, we have consistently observed that expression of the CHD4 C terminus (Δ1–1280) lowers the expression level of endogenous CHD4. One potential cause could be displacement of endogenous CHD4 from the NuRD complex, rendering it more labile.

Identification of Two Independent Repression Domains in NAB2—The results of the deletion analysis of Fig. 2 were somewhat unexpected, because NCD2, a highly conserved domain in NAB1 and NAB2, was not required for interaction with CHD4. However, these data were consistent with the previous deletion analysis of NAB1, which suggests that repression by NAB1 cannot be attributed solely to NCD2, as other C-terminal regions of NAB1 are involved (31). We tested the possibility that there are at least two independent repression domains within NAB2 by fusing either the NCD2 or the CID with the Gal4 DBD. These constructs were tested for their ability to repress the thymidine kinase promoter containing upstream Gal4 binding sites. As shown in Fig. 5, the NCD2 of NAB2 does repress, consistent with previous analysis of NAB1 (38), and the isolated CID can also repress the thymidine kinase promoter. The Gal4 DBD alone or the CID without the Gal4 DBD failed to repress the promoter. These data indicate that there are at least two separable repression domains in NAB2, one of which encompasses the CHD4 interaction domain.

CHD4 Is Required for Repression Mediated by the CID of NAB2—To test the idea that repression by the C-terminal domain of NAB2 uniquely requires CHD4, different NAB2 domains were tested for their ability to repress in the presence of dominant negative CHD4 K750C (Fig. 6*A*). To independently test the requirement for the NCD2 and the CID, we utilized deletion constructs which contained NCD1 (for interaction with Egr2) along with either NCD2 (NCD1+NCD2) or CID (NCD1+CID). Consistent with the analysis of Gal4 fusion constructs (Fig. 5), NAB2 constructs containing either NCD2 or CID were still able to repress transcription. Repression by the CID was alleviated by expression of CHD4K750C; however repression by NCD2 was unaffected by expression of either CHD4K750C (Fig. 6*A*) or the CHD4 C terminus (data not shown), indicating that CHD4 is uniquely required for repression by the CID of NAB2.

We independently tested this model by depleting endogenous CHD4 using siRNA. We assayed the ability of the CID to repress Alt. Egr2 by transfecting the NAB2 CID-containing construct in the presence of CHD4 siRNA. With siRNA-mediated depletion of endogenous CHD4, NCD1+CID was unable to repress Alt. Egr2 activity (Fig. 6*B*). We tested for nonspecific effects of the siRNA using several controls. First, a pool of non-targeting siRNA had no effect on repression by the CID of NAB2. Second, the CHD4 siRNA had no effect on repression by NCD1+NCD2 (data not shown). Finally, expression of mouse CHD4 was able to restore repression by NCD1+CID in the presence of siRNA targeted against human CHD4. Interestingly, transfection of mouse CHD4 also enhanced the ability of NCD1+CID to repress Egr2 activity. Therefore, we conclude that repression by the C-terminal end of NAB2 is dependent on its interaction with CHD4. The CHD4 siRNA had no effect on repression by full-length NAB2 (data not shown), indicating that repression by the two domains was redundant in this assay. In addition, the lack of effect might be due to partial depletion of CHD4 levels by siRNA.

Repression by the NAB2 CID Requires Histone Deacetylase Activity—The NuRD complex contains not only CHD4 but also the histone deacetylases HDAC1 and HDAC2 (43, 44, 47). If the NAB2 CID represses transcription through recruitment of CHD4, this would predict that the associated histone deacetylases in the NuRD complex are required for the repression mechanism. To test for this requirement, we repeated the repression assay in the presence of trichostatin A (TSA), an inhibitor of histone deacetylase activity. As shown in Fig. 6*C*, repression through the NAB2 CID is sensitive to the inhibitor, indicating that the

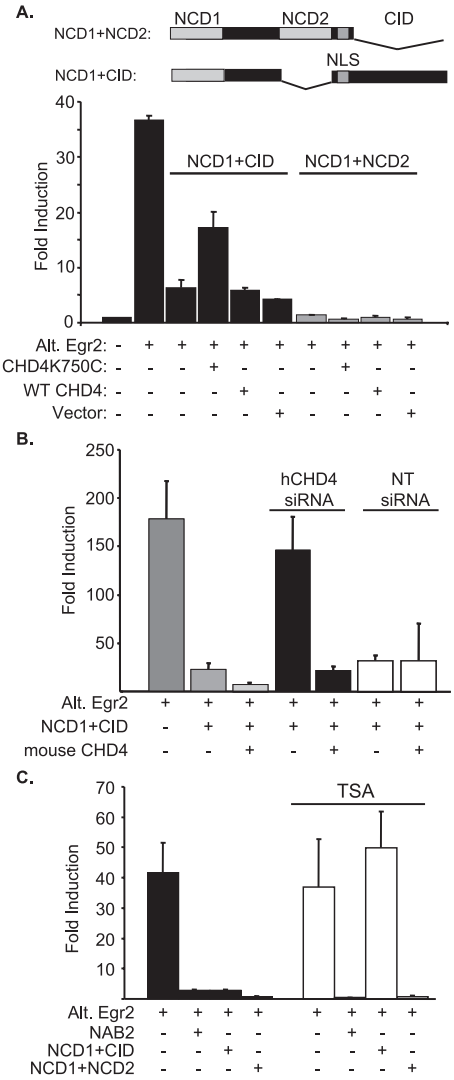

FIGURE 6. **CHD4 is required for repression by the CID of NAB2.** *A*, the diagram indicates the two NAB2 deletion constructs used for transfection assays. The extent of the deletions is the same as in Fig. 2, except that these constructs contain NCD1. JEG3 cells were transfected with the altered (*Alt.*) Egr2 reporter and 20 ng of altered Egr2. Transfections included 20 ng of the indicated NAB2 constructs: NCD1+NCD2 (*gray bars*) or NCD1+CID (*black bars*). The indicated samples were cotransfected with 200 ng of CHD4K750C, wild type (*WT*) CHD4, or vector (CMVSport6). The results are normalized to the luciferase activity of the reporter plasmid alone. Means ± S.D. of two replicate experiments are shown. *B*, JEG3 cells were transfected with altered Egr2 reporter, 20 ng of altered Egr2, and 20 ng of NCD1+CID. Indicated samples were co-transfected with either siRNA directed against human CHD4 (*black bars*) or non-targeting siRNA (*white bars*). Where indicated, cells were also transfected with 20 ng of wild type mouse CHD4. *C*, JEG3 cells were transfected with the altered Egr2 reporter, 20 ng of altered Egr2, and 20 ng of either full-length NAB2, NCD1+NCD2, or NCD1+CID. Indicated samples (*white bars*) were treated with 1 μM TSA for 24 h prior to harvest.

HDAC subunits of the NuRD complex are also required by the CID. In contrast, repression through the NCD2 domain does not require histone deacetylase activity, which again functionally distinguishes the repression mechanisms of NCD2 and CID. Repression by full-length NAB2 is not TSA-sensitive, indicating that repression by NCD2 compensates for loss of repression through the CID in this assay.

CHD4 Is Required for Repression of an Endogenous Egr2 Target Gene—Because CHD4 is part of a chromatin remodeling complex, we also tested whether CHD4 is required for repression of an endogenous EGR target gene. Previous work identified *RAD* (Ras-related gene associated with diabetes) as an Egr1 target gene (8), and this gene was also shown to be up-regulated in Schwann cells from NAB1/NAB2 knockout mice (30). Activation of the *RAD* gene by Egr2 in 293 cells was repressed by the NAB2 construct containing the CID (NCD1+CID, Fig. 7*A*). Interestingly, repression by the CID could be augmented by increased expression of CHD4. Again, use of the human CHD4 siRNA abrogated repression by the CID of NAB2, but repression was restored if mouse CHD4 was expressed in the presence of the siRNA. As an additional control, we showed that the non-targeting siRNA had little effect on repression by the CID. Furthermore, CHD4 siRNA significantly depleted (but did not eliminate) CHD4 mRNA levels (Fig. 7*B*) and did not affect CHD3 levels (data not shown). Moreover, an immunoblot using the CHD3/CHD4 antibody revealed that the total amount of CHD protein is significantly decreased by addition of the CHD4 siRNA (Fig. 7*B*), which is consistent with previous reports indicating that CHD4 is the predominant family member found in the NuRD complex (44, 46, 53).

Analysis of NAB1/NAB2 knock-out Schwann cells indicated that the *Rad* gene is repressed by NAB proteins during peripheral nerve myelination (30). Therefore, we also assayed *Rad* expression in primary rat Schwann cells. Because primary rat Schwann cells transfect very inefficiently, recombinant adenoviruses were used to express Egr2 and dominant negative NAB2, which eliminates the function of both NAB1 and NAB2 (22). Activation of endogenous *Rad* expression by Egr2 is enhanced by dominant negative NAB2, confirming that it is a NAB-regulated gene (Fig. 7*C*). Importantly, a similar derepression of *Rad* activation by Egr2 is observed with expression of dominant negative CHD4Δ1–1280 (Fig. 7*D*), indicating that repression of *Rad* expression by endogenous NAB proteins is at least partially dependent upon CHD4. Moreover, activation of *Rad* expression by Egr2 is also enhanced by TSA (Fig. 7*E*), which is consistent with previous data showing that repression through the CID is HDAC-dependent.

CHD4 Binds to an Egr2 Binding Site in the Rad Promoter—To test whether the effect of CHD4 on NAB repression was mediated directly on the *Rad* promoter, chromatin immunoprecipitation assays were used to determine whether these proteins are found colocalized with Egr2 on the *Rad* promoter. We recently adapted this technique to assay binding of Egr2 in myelinating sciatic nerve (21), composed of >80% Schwann cells. The P11 time point was chosen because it coincides with the peak of active myelination, and Egr2 and NAB proteins are maximally induced by this time point (54). Freshly dissected sciatic nerves from P11 rat pups were treated with formaldehyde to achieve cross-linking. Chromatin was then sonicated and immunoprecipitated with antibodies directed against Egr2, NAB2, CHD4, or IgG control. After reversing the cross-links, purified DNA was analyzed by quantitative PCR using primers designed to particular regions within the *Rad* locus.

Sequence analysis identified potential Egr2 binding sites at −130 and −1470 of the *Rad* promoter. However, the chromatin immunoprecipitation assay revealed that Egr2 is enriched at −130 but not at −1470 (Fig. 8), suggesting that the −130 site is the true Egr2 binding site. The results also show occupancy of both NAB2 and CHD4 at this proximal site. There is no enrichment of either protein on the −1100 bp site or the control IMG2a promoter compared with the control immunoprecipitation. Taken together, these data indicate that NAB2 and CHD4 colocalize to an Egr2 binding site 90 bp upstream of the start site within the *Rad* promoter, where they interact to repress transcription.

Interaction of NAB2 with CHD4 Is Regulated by Alternative Splicing—Characterization of the *NAB2* gene identified a splice variant that lacks exon 6 (55). Loss of exon 6 causes an in-frame deletion of a considerable portion of the CHD4-interacting domain (amino acids 426–489). We first determined that a similar alternative splicing occurs in several mouse tissues, using an RT-PCR assay (Fig. 9*A*) with primers

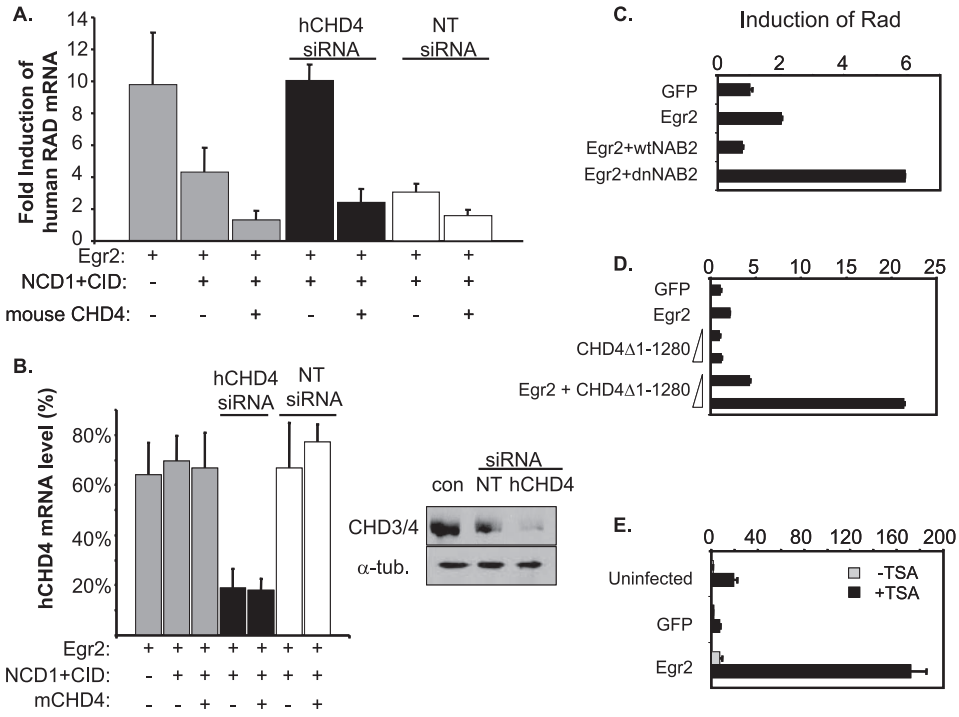

FIGURE 7. **CHD4 is required for NAB repression of endogenous Egr2 target genes.** *A*, 293 cells were transfected with 100 ng of Egr2 and, where indicated, 100 ng of NCD1+CID, 500 ng of mouse CHD4, and either non-targeting (*NT*) or human CHD4 (*hCHD4*) siRNA (62.5 pM). At 48 h after transfection, total RNA was isolated for each sample, and quantitative RT-PCR was used to determine endogenous levels of *RAD*. After normalizing to 18 S rRNA, -fold induction was determined by comparison with untreated control. Means ± S.D. represent duplicate assays in two replicate experiments. *B*, quantitative RT-PCR was used to determine endogenous levels of CHD4 in the same samples used in *A*. After normalizing to 18 S rRNA, levels of CHD4 expression were determined relative to a control culture (*con*) transfected with vector alone, which was set as 100%. Means ± S.D. were determined for duplicate measurements of two replicate experiments. The immunoblot shows lysates of 293 cells transfected with either non-targeting siRNA or siRNA (62.5 pM) directed against human CHD4. Blots were probed with antibodies directed against CHD3/CHD4 and α-tubulin (*α-tub.*) as a loading control. *C–E*, primary rat Schwann cells were infected with recombinant adenoviruses expressing the indicated proteins. After 48 h, total RNA was isolated for each sample, and quantitative RT-PCR was used to determine endogenous levels of *Rad*. After normalization to 18 S rRNA, -fold induction was determined relative to uninfected cells. Means ± S.D. represent duplicate assays, and similar results were observed in two independent experiments. In *D*, rat Schwann cells were infected with two different amounts of a recombinant adenovirus (1 or 5 × 10^9 plaque-forming units/ml) expressing CHD4Δ1–1280 in the presence or absence of the AdEgr2 virus. In *E*, Schwann cells were infected with adenovirus for 24 h, after which 1 μM TSA was added for another 24 h. *GFP*, green fluorescent protein.

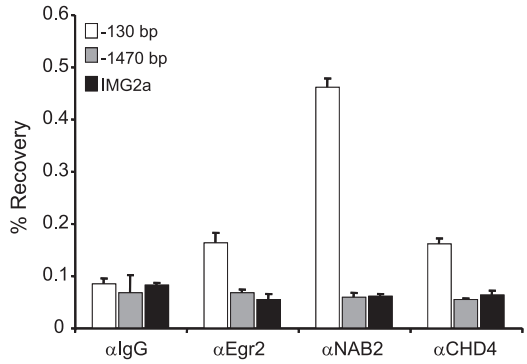

FIGURE 8. ***In vivo* recruitment of NAB2 and CHD4 to Egr2 binding sites in the *Rad* promoter.** Cross-linked chromatin was prepared from sciatic nerves of P11 rat pups and immunoprecipitated with antibodies for Egr2, Nab2, CHD3/4, or rabbit IgG (as a negative control). Purified DNA was then analyzed by quantitative PCR using primer sets targeted at potential Egr2 binding sites at −130 and −1470 relative to the *Rad* transcription start site. Occupancy is expressed as the amount of DNA recovered relative to the input sample. *IMG2a*, control immunoglobulin G2a promoter. The results are representative of three independent experiments.

flanking exon 6. In addition, several EST (expressed sequence tag) sequences corresponding to this alternatively spliced form have been identified in human sciatic nerve and mouse thymus and neurospheres (GenBank™ BQ956141, AI117547, and CX201479, respectively). We tested the ability of the NAB2 splice variant to interact with CHD4 (Fig. 9*B*). Although the expression level of NAB2Δexon6 was somewhat higher than that of wild type NAB2, CHD4 did not coimmunoprecipitate with NAB2Δexon6. We also tested whether the splice variant of NAB2 requires CHD4 activity and found that repression by this form of NAB2 was unaffected by the dominant negative CHD4K750C (Fig. 9*C*). Therefore, we have concluded that alternative splicing of NAB2 creates a CHD4-independent form of NAB2.

DISCUSSION

These results have uncovered a novel mechanism of NAB2 repression involving CHD4, which has been implicated in repression by other transcriptional repressors such as hunchback, Tramtrack69, KAP1, Ikaros, Aiolos, and FOG1 (45, 53, 56–59). Interestingly, several of these corepressors also interact with the C-terminal regions of CHD4, CHD3, and dMi2. CHD4 is an integral component of the NuRD complex, which represses transcription using both histone deacetylation and nucleosome mobilization. Both NAB1 and NAB2 interact with CHD4, and NAB2 also associates with the closely related protein CHD3, which has been identified as a component of NuRD-like complexes (43–45). The evolutionary conservation of the CHD/NAB interaction highlights the importance of this interaction for NAB repression. The mechanism of NAB repression is somewhat similar to that of the KAP1 corepressor, which interacts with the KRAB domain found in a large number of zinc finger proteins (but not in EGR proteins). A tandem PHD finger/bromodomain within KAP1 represses transcription by interacting with the CHD3 subunit of a NuRD-like complex (45). One significant difference in our study is that NAB2 interacts with both CHD3 and CHD4, whereas KAP1 interacts only with CHD3 (45).

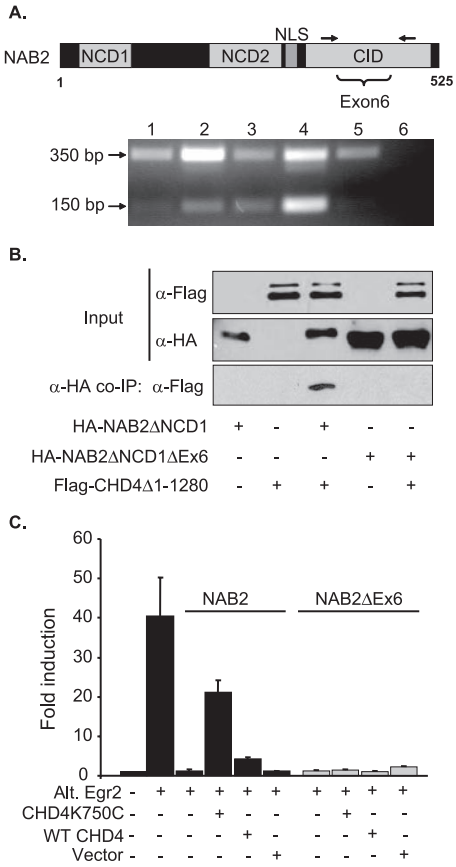

FIGURE 9. **Interaction of NAB2 with CHD4 is regulated by alternative splicing.** *A*, diagram of the NAB2 protein indicating the portion (amino acids 426–489) coded by exon 6 of the *NAB2* gene. The *short arrows* indicate primers used to detect alternative splicing of NAB2 in cDNAs prepared from the following mouse tissues: *lane 1*, liver; *lane 2*, brain; *lane 3*, T-cell; *lane 4*, pituitary; *lane 5*, testis; *lane 6*, control (no cDNA). The wild type form gives rise to a 350-bp fragment, and loss of exon 6 results in a 150-bp fragment. *B*, QT6 cells were transfected as indicated with 1 μg of 3× FLAG-tagged CHD4Δ1–1280 along with 1 μg of either NAB2ΔNCD1 or a similar NAB2 construct lacking exon 6. After immunoprecipitation of lysates with anti-HA antibody (*α-HA co-IP*), interaction with CHD proteins was assayed by immunoblotting with an anti-FLAG antibody. *Input lanes* represent 40% (*HA panel*) and 24% (*Flag panel*) of the amount of lysate used in the immunoprecipitation; all lanes are from the same exposure. *C*, JEG3 cells were transfected with altered (*Alt.*) Egr2 reporter, 20 ng of altered Egr2, and 20 ng of either NAB2 or NAB2Δexon6. The indicated samples were cotransfected with 200 ng of CHD4K750C, wild type (*WT*) CHD4, or control vector (CMVSport6). Results are normalized to the luciferase activity of the reporter plasmid alone. Means ± S.D. of two replicate experiments are shown.

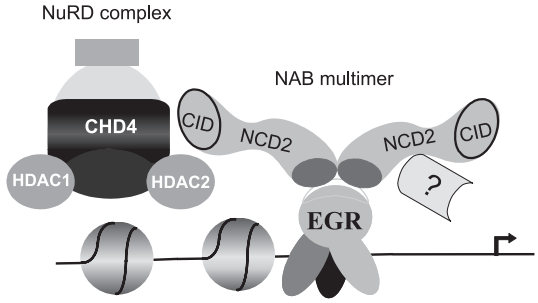

FIGURE 10. **Proposed model for the mechanism of NAB repression.** NAB proteins interact as a multimer with the NAB-binding domain of EGR proteins. The CID, C-terminal domain of NAB proteins, can recruit the NuRD complex via direct interactions with the C-terminal domain of CHD4 (or CHD3). NCD2 is an independent repression domain that exerts its effect through an as yet undefined mechanism that is HDAC-independent. NAB multimerization allows simultaneous recruitment of NuRD and other repressive complexes to a single EGR binding site.

Although several independent experiments show that repression by NAB2 CID requires CHD4, it should be noted that the NAB/CHD4 interaction is sensitive to CHD4 expression levels, because repression by the CID can be further enhanced by exogenous expression of CHD4 (Figs. 6*B* and 7*A*). Therefore, the NAB/CHD4 interaction may be a relatively weak one that facilitates NuRD recruitment to NAB-regulated promoters, which subsequently would be stabilized by the intrinsic affinity of the NuRD complex for nucleosomes (60). Accordingly, NAB proteins have not been biochemically defined as integral subunits of mammalian NuRD complexes (43, 44, 46–48), which may also reflect that they are substoichiometric compared with the abundance of CHD4 and other NuRD subunits.

Our data demonstrate that NAB2 has at least two independent repression domains. The repression mechanisms of these two domains can be distinguished because repression by the CID is uniquely affected by: (*a*) dominant negative forms of CHD4, (*b*) siRNA directed against CHD4, and (*c*) inhibition of histone deacetylase activity. The mechanism by which NCD2 represses transcription remains uncharacterized, although NCD2 bears some homology with the Dr1/NC2β transcriptional repressor (31). It was somewhat unexpected to find that NAB2 has two potentially redundant repression mechanisms, although several other repressors (*e.g.* FOG1, KAP1) also exhibit multimodal repression (45, 53).

As a result of this redundancy, the presence of NCD2 compensated for the loss of CHD4 repression in our transfection assays. However, we anticipate that endogenous NAB-regulated promoters specifically require either one or both mechanisms. Indeed, we did observe loss of NAB repression of the endogenous *Rad* gene in Schwann cells expressing dominant negative CHD4, indicating that endogenous CHD4 levels play an important role in regulation of the genomic Rad locus. Furthermore, the *in vivo* chromatin immunoprecipitation assays showed that NAB2 and CHD4 are specifically localized on an Egr2 binding site in the *Rad* promoter.

The identification of two repression domains within NAB2 has important consequences regarding the mechanism by which NAB proteins regulate EGR target promoters. Although multimerization is not required for interaction with CHD4, the multimerization status of NAB proteins, mediated by NCD1 (22), increases the diversity of complexes that can be recruited through a single EGR binding site. Our proposed model in Fig. 10 indicates that one subunit of a NAB multimer could recruit the NuRD complex, whereas the other subunit might augment repression through interaction of proteins with NCD2. Second, it is expected that differential recruitment of chromatin remodeling complexes at different EGR target promoters could significantly alter NAB activity. For example, NAB proteins do not repress all EGR target promoters equally, and in at least two promoters, NAB proteins can even augment EGR-mediated transcription (3). In this regard, it is interesting that recent evidence has suggested that CHD4 may also have positive effects on gene regulation, depending on the promoter context (61, 62). Finally, alternative splicing of NAB2 mRNA could alter regulation of EGR target genes by preventing recruitment of CHD4.

There are several physiological contexts in which the NAB/CHD connection may play an important role, including development of prostate cancer (27) and regulation of cardiac hypertrophy (63). Furthermore, recent evidence has shown that interaction of NAB corepressors with Egr2 is required for peripheral nerve myelination (28–30). Interestingly, microarray profiling has shown that CHD4 is expressed at a very high level during active myelination of peripheral nerve (54), consistent with its interaction with NAB2 in regulation of EGR2 activity. Future work will be directed toward understanding the scope and mechanism of NAB function in these contexts.

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